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Sphingolipids as biomarkers of fumonisin exposure and risk of esophageal squamous cell carcinoma in China

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Abstract

Objective: Ecologic studies of esophageal squamous cell carcinoma (ESCC) have reported an association with consumption of maize contaminated with *Fusarium verticillioides*, which produce fungal toxins referred to as fumonisins. Fumonisins disrupt sphingolipid metabolism and serum sphingolipids have been proposed as biomarkers of fumonisin exposure. We conducted a prospective nested case–control study to examine the relationship between serum sphingolipids and ESCC incidence.
Methods: Cases and controls were selected from a large prospective trial conducted in Linxian, People's Republic of China. Ninety-eight ESCC cases were randomly selected from the 639 incident ESCC ascertained during the initial 5.25 years of follow-up; 185 controls were also randomly selected based on the distribution of cases among six age and sex strata. Concentrations of sphinganine and sphingosine were determined by high-performance liquid chromatography in serum collected at the study baseline.
Results: No significant associations were found between serum sphingosine, sphinganine, or the sphinganine/sphingosine ratio and ESCC incidence in conditional and unconditional logistic regression models with adjustment for age, sex, tobacco use, and alcohol use.
Conclusion: Our study is the first prospective study to assess the relationship between sphingolipid levels, as biomarkers of fumonisin exposure, and cancer incidence. We found no significant association between sphingolipid levels and risk of ESCC.

Introduction

Epidemiologic studies have suggested a link between high consumption of maize (*Zea mays*) and wheat (*Triticum aestivum*) and esophageal cancer. Ecologic studies in China and South Africa have shown that, in those countries, communities that consume the most maize and/or wheat have elevated rates of esophageal cancer [1, 2]. Case–control and retrospective cohort studies conducted in China have also found that high maize consumption is associated with increased risk of esophageal cancer [3, 4]. In addition, three independent case–control studies have suggested a direct connection between maize consumption and esophageal cancer in northeast Italy (reviewed by Simonato *et al.* [5]). One hypothesis regarding the association between maize intake and esophageal cancer risk proposes that individuals who consume large amounts of maize are exposed to high levels of carcinogenic mycotoxins.

Maize is routinely found to be infected with *Fusarium verticillioides* (formerly *Fusarium moniliforme*), a toxigenic fungus. This fungus produces multiple families of toxic metabolites, including a recently discovered group collectively referred to as fumonisins [6]. Fumonisins have

pleiotropic effects in a variety of animal species, including equine encephalomalacia, pulmonary edema in pigs, and liver and kidney damage in most species tested [7–9].

One noted effect of fumonisins is the derangement of sphingolipid metabolism. Through the direct competitive inhibition of ceramide synthase, fumonisins increase serum and urine levels of sphingosine, sphinganine, and the sphinganine/sphingosine ratio [10, 11]. Complex sphingolipids, downstream products of ceramide synthase, are also depleted by this inhibition. Dose-dependent fumonisin-mediated alterations in serum and/or urine sphingolipids have been measured in a variety of species, including mice, rats [12], pigs [13], horses [14], and primates [15], and have been proposed for use as biomarkers of fumonisin exposure in humans [16].

Linxian is a rural county of 1 million inhabitants in Henan Province, People's Republic of China. The residents of this county have some of the highest rates of esophageal and gastric cardia cancer in the world. The age-standardized incidence rates for esophageal cancer are ~150/100,000 for men and ~125/100,000 for women, almost 10-fold higher than the national average for China [17]. A case–control study in Linxian has demonstrated that those individuals with high maize intake are at higher risk for esophageal cancer than those with low maize intake [4]. Maize in Linxian has been found to have relatively high levels of fumonisin contamination [18]. Previous reports from our group have described two large Nutrition Intervention Trials that examined the effect of vitamin and mineral supplementation on the incidence and mortality of esophageal cancer in Linxian [19–21]. We now report a nested case–control study within one of these trials, the General Population Trial, which examines the relationship between baseline serum sphingolipids, as biomarkers of fumonisin exposure, and subsequent esophageal cancer risk.

Methods and materials

Sample population and case ascertainment

A full description of the General Population Trial cohort has been published elsewhere [19, 21]. Briefly, 29,584 cancer-free individuals, aged 40–69, were recruited from the general population of four Linxian communes in 1985. All participants were interviewed to assess lifestyle, dietary patterns, medical history and were given a physical examination. Vitamin/mineral supplements were provided from March 1986 to May 1991. Active surveillance of the entire cohort during this period, including monthly contact with all subjects by village health workers and periodic review of medical records at all medical facilities in Linxian and the Cancer Hospital in the prefecture capital of Anyang, allowed complete case ascertainment with little or no loss to follow-up. Cases were reviewed by an International Endpoints Review Committee, a panel of expert cytologists, pathologists, and radiologists from the US and the PRC. This panel reviewed and confirmed 85% of the cancer diagnoses in the overall trial, including 100% of the cancer cases used in the current study. The cases in this study were diagnosed by one or more of the following methods: histology (43%), cytology (56%), or X-ray (65%). This study was approved by the Institutional Review Boards of the US National Institutes of Health and the Cancer Institute of the Chinese Academy of Medical Sciences.

During the 5.25 years of supplementation, 639 incident esophageal squamous cell carcinomas were identified in the cohort. To select participants for the nested study the cohort was divided into six sex and age strata. The six strata were defined by sex and the three age categories, $\leq$50 years, >50 and $\leq$60 years, and >60 years at the start of the intervention. From each stratum about 17 cases and 33 controls were randomly selected. A final study group of 98 cases and 185 controls was used in all analyses.

Sample collection and serum sphingolipid analysis

As part of the Nutrition Intervention Trial baseline examination, blood samples were collected from all consenting trial participants [19]. Serum was separated from whole blood by centrifugation and stored at −80 °C until aliquoted for analysis.

The samples were shipped and analyzed in a sequence designed to minimize the possible bias in the estimation of cancer risk that could be introduced if serum sphingolipid measurement varied by time or batch. Each case had controls from the same sex and age stratum within every group of 10 samples. Quality control (QC) samples were run in most batches. The QC samples consisted of aliquots of a pooled blood sample collected from a Linxian blood bank in 1995. The identity of all samples was unknown to the laboratory analysts. Serum cholesterol was measured using standard methods [22].

Sample preparation and high-performance liquid chromatography (HPLC) analyses were carried out as previously described, with minor modifications [23]. Serum was washed from vials with methanol and 200 pmol of sphinganine analog ($C_{20}S_a$) was added as an internal standard [24]. Samples were extracted with chloroform and saponified with potassium hydroxide in methanol.

Extracted sphingoid bases were then derivatized with *o*-phthalaldehyde. Samples were separated by HPLC on a C_{18} column using a methanol gradient and fluorescent detector. The absolute quantities of sphinganine and sphingosine were determined by comparing the areas under the peaks to the area under the $C_{20}S_a$ internal standard peak. All samples were analyzed in duplicate and the average of the two values was used. The QC samples included in our study had coefficients of variation (CV) for sphinganine and sphingosine of 59% and 8% respectively. The high CV for sphinganine was due to interference of unknown origin, discussed in detail below.

Statistical methods

The statistical analyses were directed towards two purposes. First, because a time trend was apparent in the sphinganine measurements over the course of our HPLC analysis, we studied the sphinganine and sphingosine measurements and their ratio to characterize and remove the observed time trend. Second, we estimated the odds of esophageal cancer as a function of the sphinganine, sphingosine, or sphinganine/sphingosine ratio measurements, adjusted for covariates for age, sex, smoking, and drinking. All of these analyses were performed in the S-Plus programming language [25]. All *p*-values were two-sided.

The sphingolipid measurements were transformed to the natural logarithmic scale for all analyses, to improve normality. Variables were constructed for the empirical quartile categories of the sphingolipid measurements where quartiles were defined using the sphingolipid measurements for the controls only. The covariate age was coded as a categorical variable with three levels ($\leq$50, >50–60, or >60 years of age), while case or control status and the covariates for sex, smoking, and drinking were coded as dichotomous variables.

Because we wished to make inference to the risk of esophageal cancer by the population quartiles of the sphingolipid levels, we used robust locally weighted regression (*lowess*) [26] to estimate the curves that represented the average trend over time in the sphinganine, sphingosine, and sphinganine/sphingosine ratio HPLC measurements. The default parameters for the *lowess* procedure in S-Plus were used, including a data fraction of 2/3, although other parameter settings were also examined. Separate curves were obtained for the cases, controls, QC samples, and the cases and controls (CC) combined. The estimated mean curves were then subtracted from the original uncorrected data to create variables for the QC corrected data and the CC corrected data. A complete description of this analysis is given in a separate study (Borkowf *et al.*, submitted).

To begin comparing sphingolipid levels between cases and controls we performed Welch's two-sample *t*-tests with unequal variances [27] to determine whether the means of the sphingosine, sphinganine, and sphinganine/sphingosine ratio measurements were the same in the two subject groups. The geometric means of these variables were computed by taking the averages of the measurements on the logarithmic scale and then back-transforming them to the original arithmetic scale, and their standard deviations were calculated by the delta method [28]. We also performed Fisher's exact tests [28] to determine whether the proportions of smoking and drinking subjects were the same in the cases and controls.

To estimate the effect of the serum sphingolipid levels on esophageal cancer incidence we performed several different regression analyses. First, conditional logistic regression [29] with the individual day of HPLC analysis as the stratum and uncorrected log-sphingolipid measurements and other baseline covariates as predictor variables were used to assess this relationship. This method adjusted for the time trend by conditioning out the effect of the day of HPLC analysis and removed the effect of this variation on estimation of the odds ratio. Next, we examined the effect of serum cholesterol on the estimate of esophageal cancer risk in our conditional logistic regression model both as a main effect and as an interaction with the sphingolipid measurements. Finally, we examined the effect of Nutrition Intervention Trial supplementation group on the estimate of esophageal cancer risk in our conditional logistic regression model both as a main effect and as an interaction with the sphingolipid measurements.

In turn, we performed unconditional logistic regressions [28] to estimate the odds of esophageal cancer as functions of the quartile categories of the sphingolipid measurements, adjusted for age, sex, smoking, and drinking. Odds ratios (ORs) were calculated with the first quartile category as the reference category, and 95% confidence intervals for these ORs were constructed on the logarithmic scale using the category-specific log-OR standard errors adjusted for the additional covariates and then back-transformed to the original scale. Likelihood ratio tests were performed to test the significance of the inclusion of the set of quartile categories. Separate models were run with the uncorrected, QC corrected, and CC corrected sphingolipid measurements.

Results

Esophageal squamous cell carcinoma cases and control subjects were divided into six categories based on sex

and age so that 16–18 cases and 33–34 controls were present in each of the strata. Case subjects comprised 48 females and 50 males, while control subjects comprised 96 females and 89 males. Tobacco use, defined as ever using tobacco for ≥ 6 months, was nearly absent among females (cases = 0%, controls = 1%). Although relatively prevalent in males (cases = 80%, controls = 64%), the median level of smoking was moderate, 10 cigarettes per day and a total accumulation of 16 pack-years. Tobacco use was not statistically different between cases and controls (chi-squared test p-value = 0.14). Alcohol use, defined as any use in the previous 12 months, showed a similar distribution, with almost no use among females (cases = 6%, controls = 4%) and more prevalent but light use among males (cases = 46%, controls = 37%). Alcohol use was also not statistically different between cases and controls (chi-square test p-value = 0.27). The lack of association between tobacco and alcohol use and case status is in general agreement with analyses of all the cases that developed in the General Population Trial [30]. In this cohort, ever using tobacco was associated with a small but significant increase in the risk of developing esophageal cancer, OR (95% CI) = 1.8 (1.4–2.4). In the same analysis no association between alcohol use and esophageal cancer was found [30].

Serum sphingolipid measurements were not normally distributed when examined on the arithmetic scale, so values were natural log transformed, which improved normality. The natural log transformed data are presented graphically in Figure 1. As expected, no time trend was observed in the sphingosine measurements. The time trend evident in the sphinganine measurements was caused by unexplained interference in the sphinganine peak, which varied over the time-course of the analytical period. The gap in analytical measurements was a trouble-shooting period. The sphinganine/sphingosine ratio reflected the time trend present in the sphinganine data (data not shown). We examined case or control status, sex, age, tobacco use, and alcohol use as potential explanatory factors for this trend using log-linear regression and analysis of covariance. None of the examined factors contributed to this variation.

To remove this HPLC time trend, all case and control sphinganine values were standardized to the QC or CC robust locally weighted regression values (*lowess*) and both methods of standardization substantially reduced the time trend. The results of these standardizations on serum sphinganine measurements are presented in Figure 1.

For each of the three sphingolipid measures, sphingosine, sphinganine, and the sphinganine/sphingosine ratio, the geometric mean of the control samples was

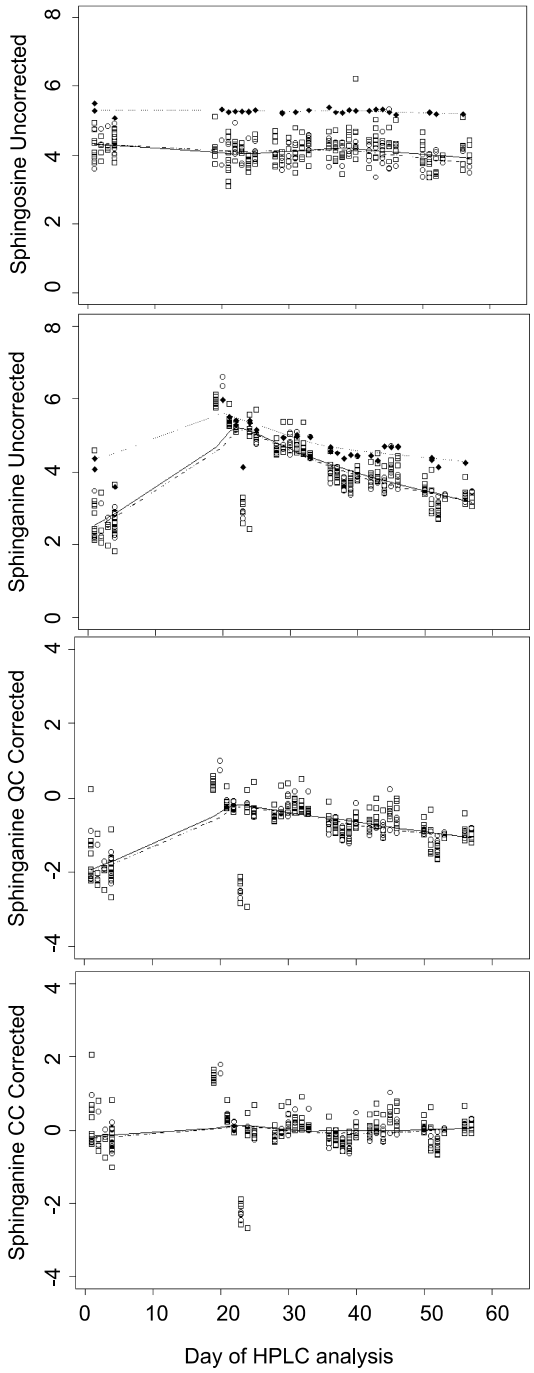

Fig. 1. Natural log of the serum sphingosine and sphinganine concentrations (nmol/L) by day of HPLC analysis. Uncorrected sphingosine and sphinganine data are presented, as well as the results after QC and CC correction of sphinganine measurements. For a description of the correction procedure see the statistical methods and results sections. Case subjects are represented by open circles (○) and dashed lines, control subjects by open squares (□) and solid lines, and QC samples by filled diamonds (◆) and dotted lines. Each point represents the mean of two measurements. Lines represent robust locally weighted regression (*lowess*) curves.

Table 1. Baseline sphingolipid measurements by case and control status

	Case subjects (n = 98), geometric mean[b] (SD)	Control subjects (n = 185), geometric mean (SD)	*p*-value[a]
Sphingosine (nmol/L)	60.7 (22.2)	63.3 (26.0)	0.38
Sphinganine (nmol/L)	48.2 (44.3)	54.6 (52.5)	0.29
Sphinganine/ sphingosine ratio	0.79 (0.75)	0.86 (0.90)	0.50

[a] *p*-values from Welch's two-sample *t*-test with unequal variances.
[b] Geometric means were exponentiated back to the arithmetic scale. Standard deviations were transformed to the arithmetic scale by the delta method.

slightly higher than that of the case samples (Table 1). Statistical comparisons of the geometric mean values for case and control subjects showed no significant differences in sphingosine, sphinganine, or the sphinganine/sphingosine ratio.

Conditional logistic regression (conditioned on day of HPLC analysis) with the uncorrected sphingolipid data was used to assess the relationship between sphingolipid levels and odds of developing esophageal cancer after controlling for age, sex, tobacco use, and alcohol use. No significant relationship was found for any of the three measures, with odds ratios (95% CI) of 0.69 (0.33–1.4) for sphingosine, 0.63 (0.29–1.4) for sphinganine, and 0.98 (0.50–2.0) for the sphinganine/sphingosine ratio.

Unconditional logistic regression analysis was used to further evaluate the relationship between serum sphingolipids, divided into empirical quartiles based solely on the controls, and risk of esophageal cancer. The model was fit for sphingosine, sphinganine, and the sphinganine/sphingosine ratio in the uncorrected, QC corrected, and CC corrected data separately, with the first quartile as the reference category. The results of multivariate unconditional logistic regression for the association between esophageal cancer incidence and serum sphingolipid lipid values after adjustment for age, sex, tobacco use, and alcohol use are presented in Table 2. No significant associations were found in the analyses of sphingosine, sphinganine, or sphinganine/sphingosine ratios.

Finally, earlier studies have suggested that fumonisin alters serum cholesterol levels [31] and cholesterol and sphingosine levels were correlated in our data set (Spearman's correlation coefficient $R = 0.37$, p-value < 0.0001). Therefore, we examined the effect of serum cholesterol on the estimate of esophageal cancer risk in our conditional logistic regression model, both as a main effect (likelihood ratio χ^2 p-value = 0.85, 0.89, and 0.85 for sphinganine, sphingosine, and sphinganine/sphingosine ratio, respectively) and as an interaction with the sphingolipid measurements (likelihood ratio χ^2 p-value = 0.53, 0.57, and 0.63). Additionally, because our subjects were selected from within a nutritional intervention trial [19], we also examined the effect of vitamin/mineral supplementation group assignment in the same conditional logistic regression model. Four variables representing assignment to the four different vitamin/mineral supplementation groups were added to the existing model and the likelihood ratio χ^2 test had p-value = 1.00 for each sphingolipid measure. When

Table 2. Adjusted[a] odds ratios (OR) and 95% confidence interval (CI) for esophageal cancer incidence by serum sphinganine, sphingosine, or sphinganine/sphingosine ratio

	Serum sphingolipid quartile – OR (95% CI)				Overall[c] *p*-value
	1[b]	2	3	4	
Sphingosine					
Uncorrected	1	1.2 (0.58–2.3)	1.09 (0.55–2.2)	0.71 (0.33–1.5)	0.61
Sphinganine					
Uncorrected	1	1.3 (0.67–2.6)	0.78 (0.37–1.6)	0.83 (0.40–1.7)	0.44
QC corrected[d]	1	0.98 (0.50–1.9)	0.72 (0.36–1.5)	0.56 (0.27–1.2)	0.36
CC corrected[d]	1	1.01 (0.52–2.0)	0.98 (0.49–1.9)	0.56 (0.26–1.2)	0.34
Sphinganine/sphingosine ratio					
Uncorrected	1	1.2 (0.61–2.4)	1.2 (0.60–2.4)	0.79 (0.37–1.7)	0.62
QC corrected	1	1.4 (0.69–2.7)	1.4 (0.67–2.7)	0.68 (0.31–1.5)	0.22
CC corrected	1	0.65 (0.32–1.3)	1.08 (0.55–2.1)	0.62 (0.30–1.3)	0.29

[a] Adjusted for age, sex, tobacco use, and alcohol use.
[b] Reference quartile.
[c] Overall *p*-value based on likelihood ratio test for inclusion of quartile categories.
[d] Two methods of correction were employed to ensure consistency of results. See statistical methods section for a full description of these corrections.

interaction terms between the supplementation group and the sphingolipid measures were added, no significant interactions were detected (likelihood ratio χ^2 p-value = 1.00 in each case).

Discussion

Intensive investigation of the extraordinary rates of esophageal squamous cell carcinoma in Linxian, China, has identified relatively few risk factors for the disease. Ecologic studies in China and South Africa have demonstrated that populations in regions with endemic esophageal cancer have both higher levels of maize consumption and higher levels of fumonisin contamination in maize than populations in areas that have lower rates of esophageal cancer [9, 18, 32]. Retrospective studies found that, during the 1970s, maize was the primary staple food for 95% of Linxian residents [3] and 60% consumed it daily [33]. A case–control study in Linxian found that individuals in the upper three quartiles of maize consumption were at higher risk for esophageal cancer when compared to those in the lowest quartile (< 24 meals per year) [4]. Very high levels of fumonisin B_1 have been found in maize from Linxian, up to 155 mg/kg [18]. Because of these studies we sought to further investigate the potential relationship between fumonisin exposure and esophageal cancer risk in Linxian.

Serum sphingolipids are elevated by fumonisin exposure in many animal species and have been proposed as biomarkers of fumonisin exposure in humans [16]. Fumonisins alter serum sphingolipids by competitively binding to the active site of the enzyme ceramide synthase [10], resulting in accumulation of the endogenous sphingolipid substrates for this enzyme. In monkeys, high doses of fumonisin B_1 have been shown to cause serum sphingolipid ratios to remain elevated for 4 weeks [34]. In rats, after an initial high dose of fumonisin B_1 exposure, urine sphingolipid ratios remained elevated with continued exposure to a relatively low dose of 1 mg/kg in the diet [12]. These studies suggest that with repeated exposure, even at low doses, human sphingolipid levels may remain elevated. The current study is the first attempt to use an individual fumonisin exposure measure, serum sphingolipids, to prospectively examine the potential association of these toxins with human esophageal cancer.

Our study showed no association between baseline serum sphingosine, sphinganine, or the sphinganine/sphingosine ratio and esophageal squamous cell carcinoma during 5.25 years of follow-up. Contrary to the predicted direction of association, control subjects had 4–13% higher, but not significantly different, levels of each of these sphingolipid measures. Conditional logistic regression of serum sphingolipid concentrations and unconditional logistic regression by quartile of sphingolipid concentrations showed no significant association between sphingolipid levels and cancer incidence. Notably, the sphinganine/sphingosine ratio, which is thought to provide the most sensitive biomarker of fumonisin exposure [13, 16], showed no significant association with esophageal cancer incidence in any of the models examined. Our results are consistent with a recently published study that reported that sphingolipid levels did not correlate with varying levels of esophageal cancer in three different communities in Africa [35]. The use of *lowess* correction and the consistency of our findings across distinct analyses leads us to conclude that the interference in our sphinganine measurements should not have altered the conclusions reached in this study.

The lack of association between serum sphingolipids and the development of esophageal cancer in our study may represent a true lack of association between fumonisin exposure and this cancer, or may reflect limitations of using serum sphingolipid elevations as a biomarker of fumonisin exposure in humans. Seasonal and annual variation in maize consumption, infection with *Fusarium* sp. and the degree of fumonisin production by *Fusarium* sp. may limit the effectiveness of serum sphingolipids as a long-term biomarker of fumonisin exposure. All of the serum samples used in this study were collected between March and May 1985, so within our study relative exposure measures should be accurate. However, it is possible that exposure during the spring of 1985 was not representative of the long-term exposure of the residents during the presumably long carcinogenic process. Another potential shortcoming of our study is that exposure, even if significant and representative, may have been too uniform across our population to detect an association with cancer incidence. The diet consumed by Linxian residents has generally been found to be uniform and monotonous [3, 33].

In addition to fumonisins, *Fusarium* sp. produce other toxic metabolites with mechanisms of action that are distinct from the sphingolipid pathway, and the high incidence of esophageal cancer in Linxian may be related to one of these additional toxins which does not affect serum sphingolipid concentrations. For example, trichothecenes (*e.g.* deoxynivalenol) and zearalenone have been found at higher levels in corn and wheat in Linxian than in Shagqiu, a low-risk area for esophageal cancer in Henan Province [36]. Another potential agent in this association is the as-yet-unidentified agent in *F. verticillioides* culture material that has been shown to form DNA adducts *in vitro* [37]. In addition,

nutritional deficiencies in niacin, zinc, and other nutrients can occur in individuals with high rates of maize consumption, and these deficiencies may also play a role in the development of esophageal cancer [38].

Complete validation of serum and/or urine sphingolipid levels as biomarkers of human fumonisin exposure is an important priority being undertaken by other research groups [16]. In addition, the development of other potential biomarkers, such as free fumonisin in the urine or the recently identified DNA adduct mentioned above, may be required before definitive studies can be completed to determine if fumonisins or other mycotoxins are etiologic agents in the development of esophageal cancer.

In summary, elevated sphingolipid levels, as biomarkers of fumonisin exposure, were not associated with an increased risk of esophageal squamous cell carcinoma in this prospective study.

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